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Review

Molecular mechanisms of the circadian clockwork in mammals



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ABSTRACT

Circadian rhythms enable organisms to co-ordinate biological processes with the predictable 24 h cycle of day and night. Given that molecular clocks that coordinate such biological timing have evolved in almost all organisms, it is clear that being synchronous with the external environment confers competitive advantage. Conversely, it is apparent that being out of phase is detrimental, resulting in a number of clinical conditions, many of which are linked to metabolic dysfunction. The canonical clockwork involves a core set of genes that negatively regulate themselves through a so-called transcription translation feedback loop. However, recent studies describing evolutionarily conserved oscillations in redox reactions link circadian rhythms to metabolic processes, and in particular, redox pathways. In this review we describe the evidence for the interaction between transcriptional loops, redox and metabolism in mammals and suggest the clock may be potential target for the treatment of disease.

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1. Introduction

Almost all organisms display behavioural and biochemical oscillations over a 24 h period. These circadian (Latin; *circa*, “approximately”, *-diem*, “day”) rhythms are evolutionarily conserved and driven by the need to synchronise biological activity with the ever-changing, but predictable, environment of the rotating Earth. In mammals processes as diverse as temperature and blood pressure fluctuations, sleep–wake cycles, and glucose and lipid metabolism, are all under rhythmic control (Fig. 1). Circadian rhythms are not merely stimulus-evoked responses since they persist in the absence of external cues, they can adjust to local time (that is, they ‘entrain’ to cues) and the period and amplitude of the rhythm is not affected by steady-state temperature, a phenomenon known as ‘temperature-compensation’. As a result of circadian rhythm organisms are able to temporally segregate incompatible or competing processes and synchronise periods of activity, feeding and rest with the solar day [1,2].

This ability to pre-empt environmental changes has been shown to confer selective advantage to organisms. For example, cyanobacteria oscillating with endogenous periods matched to an externally imposed 24 h light–dark cycle reproduce far better than colonies that are asynchronous [3]. Similarly, *Arabidopsis thaliana*

plants with rhythms matched to the external light cycle grow faster than mismatched ones [4], and wild type *Drosophila melanogaster* display greater reproductive fitness than counterparts with circadian clock mutations [5].

While being synchronous is advantageous, it follows that being out of phase with the external environment may be detrimental. Indeed, dysfunction of circadian rhythms as a result of shift work or neurodegenerative disorders, for example, has been linked to a wide range of human disease states including depression, pain, inflammation, heart disease, diabetes and cancer [6–11] indicating the importance of keeping time. Additionally, circadian clocks can be used to our benefit when treating patients with medicines, as exemplified by the fact that time of dosing significantly affects both the efficacy and side-effect profile of drug treatments [12–14]. Most notably, dosing non-steroidal anti-inflammatory drugs (NSAIDs) and statins in the evening reduces toxicity and improves their efficacy [13,15]. Furthermore, baseline circadian rhythmicity prior to chemotherapy has been reported to correlate with therapeutic outcome and survival rates of patients with metastatic colorectal cancer, indicating that patients with perturbed rhythms may respond less favourable [16]. In line with this, a recent clinical study suggests tolerability of chemotherapy regimens in advanced cancer patients could be improved by maintenance of circadian rhythm following treatment [17]. Therefore, a better understanding of the circadian clock could have far reaching implications for human health and for the treatment of pathological states.

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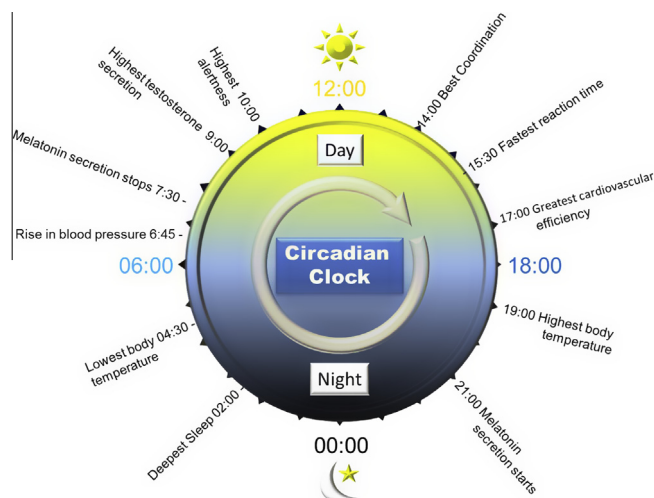


Fig. 1. Schematic representation of key physiological changes over the circadian day. Representation of circadian changes in human physiology (adapted from [112]).

2. Central and peripheral clocks

In mammals the circadian clock is arranged in a hierarchical manner with a central clock in the brain maintaining the daily rhythm in accordance with the external environment. This timing information is then outputted to the periphery to synchronise the body's tissue clocks. In mammals the paired suprachiasmatic nuclei of the hypothalamus (SCN) are the master pacemaker, and the SCN act as a coordinator for the rest of the body. The SCN are reset every day by light on the retina, and dedicated monosynaptic projections terminate in the SCN resulting in activation of various signalling pathways including transcription of clock genes and chromatin remodelling [18].

Metabolic activity in the SCN is high during the daytime and low at night. However, at night the metabolic activity can be increased by acute exposure to light. Light is the major Zeitgeber ('time giver') in humans and is capable of altering the circadian phase by up to an hour [19]. Having periods of light and dark is important for normal physiology. Preterm infants exposed to a light/dark cycle in intensive care units gain weight faster than those under traditional continuous light conditions which may be a result of decreased metabolic demand [20]. Lesions of the SCN abolish circadian rhythms in drinking and locomotor activity in rodents [21,22]. Transplantation studies, where intact SCN are transferred into lesioned animals showed that rhythms in locomotor activity could be re-established and that a soluble factor was likely to be required for this restoration, rather than the formation of new neural connections [23]. It was subsequently shown that epidermal growth factor signalling was a potential candidate for modulating locomotor activity, since levels of transforming growth factor- α (TGF- α) are rhythmic and exogenous TGF- α inhibited activity [24].

While the SCN ensure that peripheral clocks are appropriately synchronised, peripheral tissues oscillate independently of the SCN. Indeed, isolated tissues exhibit rhythms for several days, and even weeks, *ex vivo* [25–27]. In addition, by restricting food availability in mice, circadian gene expression in the liver can desynchronise from that in SCN with a phase shift of up to 12 h [28,29]. In effect, the clock in the liver and that in the SCN are on opposite cycles. It is likely that this can lead to deleterious consequences when oscillations between central and peripheral clocks are mismatched. Conversely, restoration of circadian behaviour by the use of restricted feeding regimes can diminish metabolic

abnormalities in the liver, as has been demonstrated, for example, in a mouse model of Huntington's disease, whose circadian rhythms are disrupted as the disease progresses [30]. This underlines the functional link between feeding, and therefore metabolism, and circadian rhythmicity.

3. The transcription-translation feedback loop (TTFL) model

Key components of the molecular clock that drive rhythmic behaviour have been identified and characterised in numerous species including flies, molluscs, fish and mammals [31–33]. While there are significant differences in their machinery, the logic appears to be similar, i.e. transcription factors positively induce 'clock genes' which then negatively feedback, progressively inhibiting transcription, thus forming a 'transcription-translation feedback loop' (TTFL) [32,34]. In addition, the cycling clock genes of the TTFL induce numerous other output genes thereby enabling the timing of cellular processes.

In mammals, the master transcription factors BMAL1/2 (brain and muscle Arnt-like protein) dimerize with CLOCK (circadian locomotor output cycles kaput), or NPAS2 (Neuronal PAS domain-containing protein 2) in the brain [35,36] which drives the transcription of *Period* (*Per1–3*) and *Cryptochrome* (*Cry1/2*) genes via interactions with E-box promoter elements [37]. PER and CRY proteins in turn form complexes in the cytoplasm, and at a certain threshold, the PER/CRY complex migrates to the nucleus where it inhibits the action of BMAL and CLOCK, and thus PER and CRY transcription [38–40]. The inhibitory PER/CRY complexes are subsequently degraded in the proteasome following phosphorylation by casein kinase 1 ϵ (CK1 ϵ) [41] and then ubiquitination [42] which removes the inhibition on CLOCK and BMAL, allowing the feedback loop to restart again in a 24 h loop.

The system is further fine-tuned by complex interactions with numerous other intertwined feedback loops. Critically, the retinoic acid receptor-related orphan receptors, REV-ERB α/β and ROR α bind to enhancer elements on the *Bmal1* promoter to inhibit or promote transcription, respectively [43]. Oscillations in transcription of REV-ERB α and ROR α drive the rhythmic expression of BMAL1, and the BMAL1/CLOCK complex acts directly on the REV-ERB α gene, driving an 'accessory' loop (Fig. 2).

4. Genome-wide circadian gene expression

An additional layer of regulation is provided by genome-level sculpting of gene expression at both the level of epigenetics and transcription. Epigenetic control plays a role in fine tuning the circadian clock, and accordingly, histone modification has been shown to be an integral part of the TTFL [44,45]. PER/CRY multimers, for example, recruit a PSF/Sin3-HDAC protein complex which inhibits transcription of deacetylating histones 3 and 4 thereby altering downstream gene expression [46]. It has also recently been shown that circadian gene oscillation can be abolished in *A. thaliana* by inhibition of histone acetylation and trimethylation [47] indicating the importance of post-translational modifications on clock gene activation.

Microarray experiments (and more recently RNA sequencing approaches) have been useful in elucidating the temporal nature of global gene expression. A range of studies have shown that a significant proportion of genes are expressed in a circadian manner and that there are tissue-specific variations in their expression pattern. It has been reported that 7–21% active genes oscillate in liver and adipose tissue [48]. However, expression patterns differ between tissues. For example, in the heart, genes are expressed in a synchronous manner with similar peak expression profiles, while in the liver, genes showed a broad distribution of expression

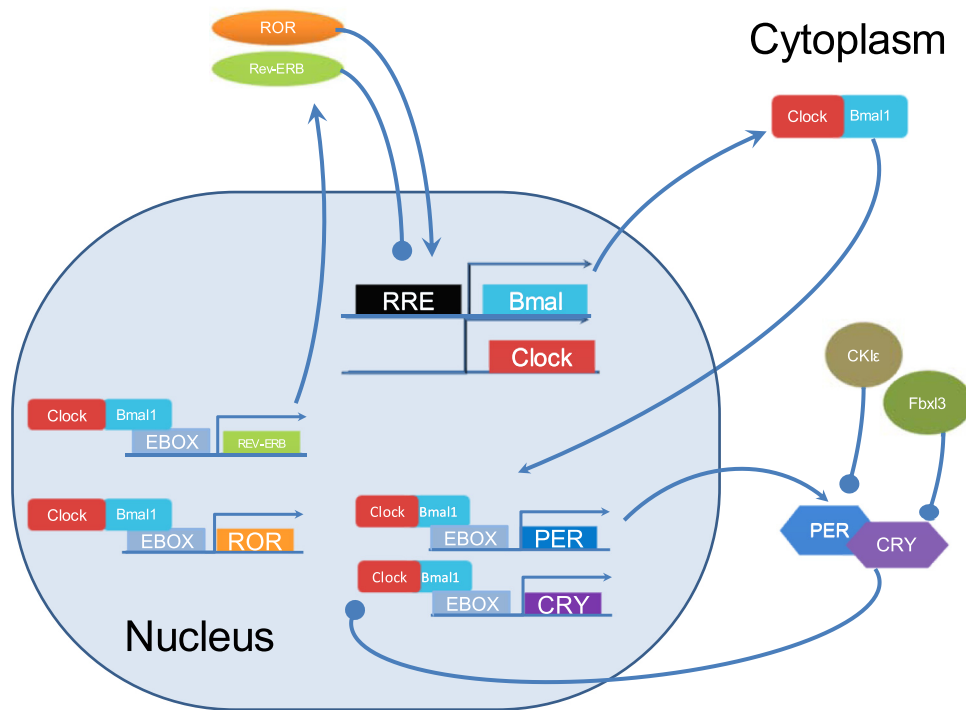


Fig. 2. Schematic representation of the core circadian clock. BMAL and CLOCK transcription products translocate to the cytoplasm and dimerize. They then return to the nucleus binding to E-box regions and promoting Period (PER), Cryptochrome (CRY), REV-ERB and ROR transcription. PER and CRY translocate to the cytoplasm, dimerize and return to the nucleus inhibiting the binding of the CLOCK/BMAL complex. PER and CRY are subsequently degraded following binding to casein kinase I ϵ (CKI ϵ) and Fbxl3 respectively. REV-ERB inhibits transcription of *Bmal1* whilst ROR promotes *Bmal1* expression.

over the circadian day [49]. Additionally, the transcripts that are under circadian control differ between tissues. Of the 650 genes that were determined to be expressed rhythmically in the SCN and liver, only 28 were found to overlap between tissues [50]. Such studies are also useful in elucidating the interaction between circadian genes. This is illustrated by the fact that the links to REV-ERB α and ROR in the clockwork were no doubt accelerated by the discovery that the REV-ERB α /ROR response elements generate gene expression in phase with *Bmal1* and antiphase with *Per2* in both the SCN and liver [51]. Recent studies using genome-wide cistromic assays have shown ROR γ directly regulates metabolic gene transcription and mice deficient in ROR γ display improved insulin sensitivity and glucose tolerance [52]. This suggests antagonist for ROR γ may be useful for the treatment of metabolic disease.

5. Nuclear receptors

It should be noted that REV-ERB and ROR are not the only nuclear receptors that are expressed in a circadian manner. Indeed, more than 50% of nuclear receptors are rhythmically expressed [53]. Since nuclear receptors usually modulate transcription, they are likely involved in the circadian expression of their target genes. For example, glucocorticoid receptors (GR) interacting with CRY1 and CRY2 are involved in repression of glucocorticoid synthesis [54] and low levels of corticosteroid are able to enhance expression of *Per1* via GR [55].

The clinically-important PPAR (peroxisome proliferator-activated receptors) family of nuclear receptors are also under clock regulation. All three isoforms of PPAR display rhythmic expression, and PPAR α is a direct target for CLOCK and BMAL1 [53,56,57]. Furthermore, the rhythmic expression of liver PPAR α is disrupted in *Clock* and *Bmal1* mutant mice [58] and reciprocally, PPAR α knock-out mice display altered hepatic *Bmal1* and *Per3* rhythms. This is of major interest since PPAR α and PPAR γ ligands, such as clofibrate and rosiglitazone, are currently used clinically to reduce lipid

levels and improve glycaemic control, respectively. Such metabolic disorders have been linked independently to circadian disruption, but an open question is whether these drugs exert their effect via modulation of the circadian clockwork. However, there is growing interest in targeting nuclear receptors for clinical indications and new compounds could, at the very least, be useful pharmacological tools to tease apart the interactions between the clock and nuclear receptors [59,60].

6. Post-translational modifications

Post-transcriptional and post-translational modifications (such as phosphorylation, acetylation, methylation, SUMOylation and ubiquitination) are integral to the molecular mechanism of the clock [61–63]. Indeed, it is likely that post-transcriptional processes play a significant role in driving tissue-specific circadian programmes [2,64–67]. Within the ‘core’ clock machinery itself, phosphorylation of PER2 promotes complex formation with CRY which in turn promotes nuclear translocation [68]. Additionally, phosphorylation of PER1 mediates cellular location [69]. BMAL1 displays rhythmic conjugation to SUMO2/3 (Small Ubiquitin-like Modifier 2/3), with levels of the complex directly proportional to transcriptional activity [62]. SUMOylation of BMAL1 also promotes nuclear localisation as well as ubiquitin-dependent degradation. In the mouse liver, SUMOylation of BMAL1 displays a circadian rhythm that coincides with its activation [61].

Protein degradation following phosphorylation is an important timing element of the clock. This is exemplified by the fact that CRY1/2 degradation is modulated by Fbxl3, an F-box protein with 3 leucine repeats [70]. Mutations in Fbxl3, that reduce the affinity for CRY, result in mice with a prolonged circadian period of around 27 h. Similarly, PER2 is hyperphosphorylated by casein kinase I ϵ (CKI ϵ) [41]. Mutations in CKI ϵ , or the phosphorylation site of PER2 [71], result in decreased degradation of PER2. This presents in the clinic as familial advanced sleep phase syndrome (FASPS) a

circadian disruption disorder where patients display advanced sleep onset (18:00–20:00) and early awakening (03:00) [41,71]. Interestingly, sleep disturbance is also a common complaint in several neurodegenerative diseases [72] with recent studies implicating a role of clock genes; Alterations in Bmal1 expression profiles have been observed in patients with Parkinson's [73] and polymorphisms in CLOCK has been shown to be a risk factor for the development Alzheimer's [74,75].

7. Redox and the clock

The possible interplay between redox state and circadian rhythm has been long known but it was unclear until recently whether redox oscillations are a driver of the clock, are an accessory loop, or a biomarker of cellular time. The discovery that circadian redox oscillations appear to be conserved throughout evolution, and that circadian oscillations in redox parameters persist in the absence of transcriptional apparatus, has strengthened the fundamental link between metabolic processes and the molecular clock [76–78].

Circadian rhythms have been observed in cyanobacteria *Synechococcus elongatus* in the absence of transcription and translation [79]. Three proteins, KaiA, KaiB and KaiC, undergo rhythmic changes in phosphorylation state [80]. Remarkably, an in vitro clock can be created; with just these three proteins and ATP as an energy source phosphorylation of KaiC cycles over a 24 h period [81]. As cyanobacteria are photosynthetic they need to maintain synchronicity to the external light environment. In photosynthetic organisms the redox state of quinone compounds varies in response to light. It was discovered that the protein LdpA senses the redox state of the cell which is reduced in high light conditions and increased in low light. In redox dependent manner LdpA modulates levels of CikA which associates with and alters the phosphorylation state of KaiA which in turn alters the rhythm of the cell to fit external cues [82,83]. Indeed, artificially altering the redox state of the cell by addition of quinone compounds can alter and/or reset the phase of *Synechococcus* cultures in a similar manner to altering light levels.

Similarly, in zebrafish light exposure alters redox state, which increases expression of *Cry1* and *Per1* orthologues, which is in turn reciprocally related to catalase expression. This further underscores the role of hydrogen peroxide (H_2O_2) as an intermediate signal between light and gene expression [84].

In mammals, oscillations in FAD and NADP redox state have been observed in SCN tissue slices, and these were able to alter the excitability of SCN neurons through non-transcriptional modulation of membrane potassium channels [85]. Additionally, reducing extracellular potassium and calcium inhibits *Per1* expression in the mouse SCN [86,87] suggesting that redox plays a role in neuronal excitability. The fact that cycling of redox state may have functional effects is intriguing and requires further study to determine if such oscillations drive, or are driven by, circadian rhythms.

Approximately ten thousand genes are known to be under circadian control in mammals [2,49–51]. Numerous studies have investigated circadian oscillations in gene expression in key metabolic tissues, including brown fat, liver and skeletal muscle [2,51,88]. Several key metabolic enzymes are rhythmically expressed in the liver, for example, including succinate dehydrogenase 1, ketohexokinase, aldolase 2, enolase 1 and aconitase [64]. Since numerous metabolic diseases appear to have a circadian-related dysfunction [7], it follows that core cellular metabolism and the clock are intimately connected.

Metabolic processes alter the redox balance of cells by producing oxidants and depleting reducing agents. Due to the potential damaging nature of oxidants, there are numerous mechanisms that

maintain homeostasis. Large quantities of hydrogen peroxide are produced in the endoplasmic reticulum – one molecule of H_2O_2 is generated for each disulphide bond formed during protein folding [89,90], and large quantities of H_2O_2 are produced during mitochondrial metabolic processes, particularly because of electron transport leakage releasing O_2^- radicals. In addition, cytokines and growth factors stimulate H_2O_2 production via NADPH oxidase, which is used as a second messenger [91]. While hydrogen peroxide is a key cytotoxic component of phagocytic immune cell activity it is also able to modify proteins, lipids and nucleic acids. Despite the significant quantities produced under normal physiological conditions, ROS (reactive oxygen species) are rapidly detoxified by scavengers, thus preventing oxidative stress. ROS are removed by a variety of enzymes such as catalase, SOD (superoxide dismutase), glutathione peroxidase and peroxiredoxins, as well as by Vitamin C and E, carotenoids and lipoic acid [92–94]. Imbalance in ROS homeostatic mechanisms has been implicated in neurodegenerative conditions such as Parkinson's and Alzheimer's disease [93].

Another endogenous antioxidant of circadian interest is the hormone melatonin which is secreted from the pineal gland with levels increasing dramatically during the dark, regardless of an organisms behavioural pattern [95,96]. In diurnal animals melatonin may promote sleep, while in nocturnal animals levels are correlated with wakefulness. Melatonin has been proposed to be an antioxidant and is able to scavenge free radicals [97,98], and also stimulates antioxidant enzymes [99]. In 3T3-L1 pre-adipocytes, for example, melatonin was shown to enhance the activities of SOD, as well as glutathione peroxidase and glutathione reductase in a manner dependent on melatonin receptor 2 [100]. Recently it has been shown that shift workers have reduced melatonin levels and altered secretion profiles which may lead to increased risk of cancer [101]. Additionally, melatonin levels are reduced in patients with type 1 diabetes which may be a result of high glucose concentration altering melatonin production as is seen in diabetic rats [102].

The redox state of a cell has been shown to be an integral component in the regulation of the molecular clock. The DNA binding of NPAS2 and CLOCK is influenced by the redox state of NAD(H) and NADP(H) as is the NPAS2:BMAL1 heterodimer [103]. NADP inhibits NPAS2:BMAL1 binding to DNA, in contrast, the NADPH promotes DNA binding. At a ratio of NADP:NADP(H) of over 75% NPAS2:BMAL1 DNA binding increases sharply whereas below 75% binding is reduced. This suggests that the metabolic (redox) state of a cell could directly influence the core circadian clock.

8. Peroxiredoxins

Peroxiredoxins (PRDXs) are a family of antioxidants that help to prevent cellular damage resulting from the production of reactive oxygen species [104–106]. There are six PRDX isoforms (PRDX 1–6) with differing cellular localisation: cytosol (1, 2, 5 and 6) mitochondria (3 and 5), peroxisome (5) and the endoplasmic reticulum (4) [105,107,108].

PRDXs have a redox active cysteine residue that binds H_2O_2 forming a sulphenic acid. In most cases, a homodimer is formed by a resolving cysteine at the C-terminus forming a disulphide bond with the cysteine sulphenic acid. This bond can then be 'resolved' by thioredoxin, enabling further catabolism of peroxide by PRDX. Additionally, the 2-Cys peroxiredoxins (1–4) can be over-oxidised to sulphinic and sulphonic acids ($-SO_{2/3}H$) with increasing H_2O_2 concentration. Overoxidation stabilises the formation of inactivated pentamers, in effect decamer structures. The decamer may then be recycled to the active monomer by sulphiredoxin in an ATP-dependent reaction [92,109,110] (Fig. 3). This cycling of redox

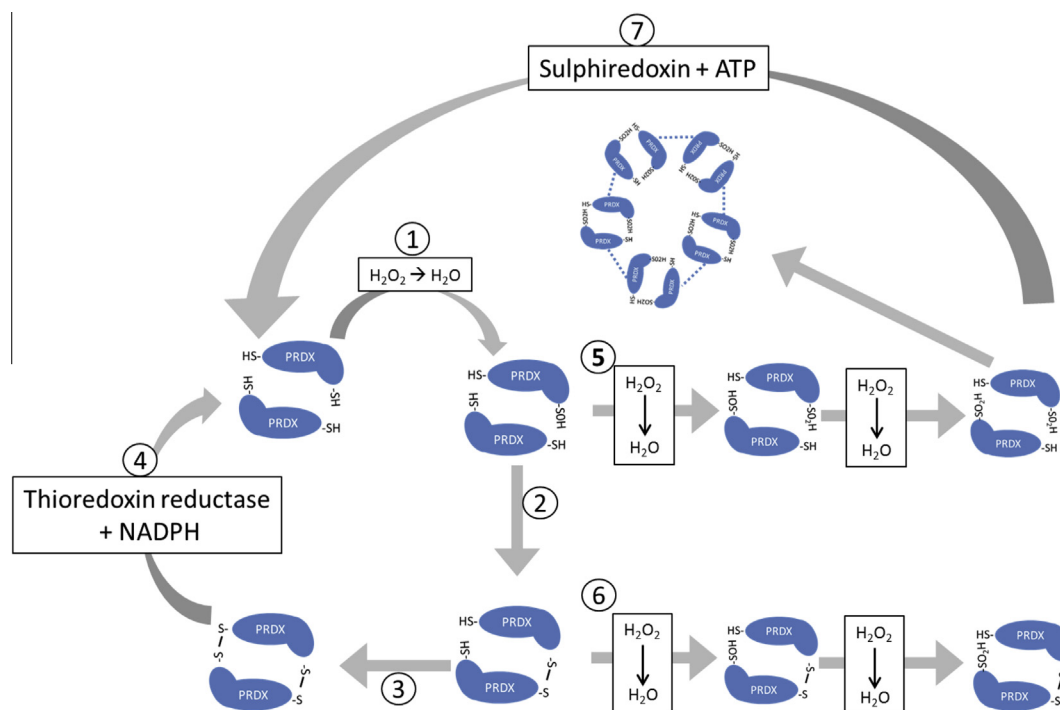


Fig. 3. Schematic representation of the 2-Cys peroxiredoxin reaction with H₂O₂. (1) PRDX binds H₂O₂ forming a sulphenic acid. (2) A homodimer is formed by a resolving cysteine at the C-terminus forming one, and then two (3) disulphide bonds, with the cysteine sulphenic acid. (4) Thioredoxin resolves the disulphide bond enabling further catabolism of peroxide by PRDX. (5) + (6) Increasing H₂O₂ concentration causes 2-Cys peroxiredoxins (I–IV) to be hyperoxidized to sulphinic and sulphonic forms, resulting in overoxidized monomers and dimers. The overoxidized monomers potentially form a decameric structure. (7) Sulphiredoxin recycles PRDX to monomeric forms.

state of PRDX had been shown to follow a circadian rhythm [64], with two forms of PRDX6 oscillating in antiphase in the liver, emphasising a role of post-translational modification in circadian rhythmicity. PRDX2 oscillations have also been shown in the nucleus of keratinocytes with knockdown of PRDX2 resulting in increased intracellular ROS and induction of PRDX3 [111].

While the core clockwork utilises transcription-translation feedback loops (TTFLs), the numerous post-translational modifications outlined above, coupled to the fact that circadian oscillations can occur in a simple *in vitro* system [81], suggests transcription is not required for the induction and maintenance of circadian rhythms. To assess this, oscillation in redox state of PRDX was used as a marker of circadian rhythmicity in isolated human red blood cells. Using western blotting, it was shown that the redox cycle of PRDXs was circadian, temperature-compensated and able to be reset to the external environment [77]. Since red blood cells in mammals are anucleate, and thus devoid of transcriptional activity, it follows that TTFLs cannot be solely responsible for the maintenance of the circadian rhythms in complex organisms such as humans. The redox state of PRDXs is driven by the metabolic state of the cell, but is independent of transcription, which suggests metabolism itself is also a key driver of circadian rhythm.

9. Concluding remarks

Circadian rhythms are all pervading, being responsible for myriad biochemical, cellular and behavioural processes. The importance of keeping in time with the changing environment is evident from the numerous mechanisms that have evolved to keep track of time. As organisms became more complex and diverged, so too did the molecular clock. It now appears that cycling of redox state is central to the development of circadian timing [76]. Therefore, whilst TTFLs are indeed important in the maintenance of complex circadian phenotypes, and generating transcriptional

programmes at a genome scale, metabolism itself is likely to be at the core of circadian rhythm generation. Regardless of the details of the intrinsic molecular machinery that generates 24 h timing, it is clear that being synchronous with the environment confers biological advantage, while being out of synchrony poses problems. It is also apparent that unravelling the molecular intricacies of the clockwork could give rise to potential drug targets and may lead to novel treatments of pervasive diseases, including cancer, diabetes, obesity and neurodegeneration.

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